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Generation of Chemotactic Factors by *Rhizopus oryzae* in the Presence and Absence of Serum: Relationship to Hyphal Damage Mediated by Human Neutrophils and Effects of Hyperglycemia and Ketoacidosis

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As our previous studies had shown that human neutrophils could kill *Rhizopus oryzae* hyphae in vitro, interactions of these hyphae with neutrophils and serum were further explored. Heated or fresh normal human sera suppressed hyphal metabolic activity as determined by [^{14}C]uracil uptake, but severe ketoacidosis (8×10^{-3} M β-hydroxybutyric acid plus 2×10^{-3} M acetoacetic acid at pH 7.0) negated this effect. Hyperglycemia (500 mg/dl) and severe ketoacidosis did not affect damage to hyphae by human neutrophils. Hyphae generated factors from sera which induced comparable chemotactic responses by neutrophils obtained from both normal and diabetic subjects, using a leading front assay performed in modified Boyden chambers. Zymosan-stimulated neutrophil chemotaxis was marginally depressed only by the combined elevation of both glucose (500 mg/dl) and ketoacids (10^{-2} M) irrespective of pH (7.0 to 7.4), but not by any of these factors alone. Protein-containing supernatants from live or killed hyphae were chemotactic in the absence of serum based upon "checkerboard" assays varying the concentrations of hyphal supernatants above and below filters in the Boyden chambers. The supernatant-induced chemotactic response by neutrophils from diabetic subjects was minimally less than that of normal neutrophils ($P < 0.05$). These findings indicate that *R. oryzae* hyphae can generate chemotactic factors which might prove to influence the inflammatory response to infections in vivo, and that severe hyperglycemia and ketoacidosis might affect interaction between the host and invading hyphae in mucormycosis.

Rhizopus oryzae, the commonest cause of mucormycosis, causes infections primarily associated with poorly controlled diabetes mellitus in humans and experimental animals (16). Human hosts are capable of clearing invasive hyphae from tissues, as mucormycosis has been cured by improved diabetic regulation alone (4) or by treatment with only subinhibitory or fungistatic levels of antifungal agents (1, 5). Neutrophils appear to be a major component of these early host defense mechanisms, as neutropenia has been associated with some cases of mucormycosis in humans (17), and neutrophil-rich exudates surround hyphal elements in experimentally infected normal rabbits in which infections clear, but neutrophil responses are depressed in ketoacidotic rabbits which develop fulminant mucormycosis (6, 7). In previous studies, we demonstrated that neutrophils could damage and probably kill *R. oryzae* hyphae in vitro despite the large size of the hyphae, which precluded complete ingestion by phagocytic cells (11). In other laboratories, delayed or diminished chemotactic responses to a variety of nonspecific stimulants have been observed in vivo and in vitro in diabetic humans and experimental animals and have been postulated as a cause of an observed increased incidence of infections (8, 15, 18–20, 22). Other studies have documented a variety of diabetes-associated defects in neutrophil adherence and phagocytic and bactericidal function (2, 3, 9, 13, 21, 25), though methodologies and specific results have varied among different laboratories. Diabetic ketoacidosis may also affect hyphal viability, as Gale and Welch observed inhibition of growth of *R. oryzae* spores in normal but not in ketoacidotic serum (14). Therefore, we decided to study directly the effects of hyperglycemia and ketoacidosis on the interactions of *R. oryzae* hyphae with human neutrophils and serum in vitro, including serum and neutrophil-dependent antihyphal mecha-

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nisms, as well as hyphal generation of chemotactic factors.

MATERIALS AND METHODS

Neutrophils and sera. Clotted blood for serum and heparinized blood were obtained from healthy normal subjects and from eight patients hospitalized with poorly controlled diabetes mellitus (blood glucose 218 to 538 mg/dl; two patients with pH 7.20 to 7.28 and ketonemia, six with pH ≥7.35 and no ketonemia). Neutrophils were obtained as in previous studies (11) by dextran sedimentation after separation on a Hypaque-Ficoll gradient, and erythrocytes were lysed by incubation in Tris-buffered ammonium chloride (Boyle solution).

Hyphae. As in previous studies (11), an isolate of *R. oryzae* originally obtained from a patient with mucormycosis was maintained on Sabouraud agar (Emmons modified) slants and subcultured for 2 to 5 days before harvesting. After removal of hyphae by filtration through cheesecloth (11), washed spores were diluted to 2.0×10^5/ml in Sabouraud broth (or Eagle minimal essential medium supplemented with nonessential amino acids), incubated at 37°C for 5 to 8 h until ≥90% of spores had germinated to ≥30 μm in length, stored at 4°C overnight, and washed in Hanks balanced salt solution or Gey medium before interaction with neutrophils or serum.

For production of supernatants used in chemotactic studies, hyphae were washed and suspended in sterile, deionized water or 0.5 M NH_4HCO_3 (pH 8.0) buffer. In some experiments, live hyphae were placed in Sabouraud or yeast-nitrogen base broth (supplemented with asparagine and glucose) or in Eagle medium and rotated overnight for 18 h at room temperature. Hyphae were removed by centrifugation, and supernatants from live organisms were retained. Other hyphae were placed in glass petri dishes (10 by 100 mm) and exposed to UV (model 782L 20 germicidal lamp, Atlantic Ultraviolet Corp., Bay Shore, N.Y.) at 15 cm for 18 h. Thereafter, supernatants were separated from hyphae, frozen, lyophilized, and stored at −20°C until ready for use. The protein content of supernatants was quantitated by the Lowry technique or by quantitation of primary amines using fluorescamine, and carbohydrate by the anthrone test as in our previous studies (12).

Metabolic activity of hyphae; hyphal damage. Experiments to determine metabolic activity of and damage to hyphae were performed as in previous studies (11) with the following modifications. Uptake of [^{14}C]uracil was measured after 1 h of incubation in Hanks balanced salt solution containing graded amounts (10 to 80%) of human serum. Incubations with leukocytes contained 0.5 ml per tube with 1.0×10^6 neutrophils and 1.0×10^5 hyphae with or without sera, and neutrophils were added to control tubes after the 1-h incubations at 37°C, at the time of lysis of neutrophils with distilled water. Washed hyphae were then incubated for 2 h at 30°C with 2.0 μCi of [^{14}C]uracil, washed of unbound isotope, and collected on filters for liquid scintillation counting, using an automated multiple-sample harvester as in previous studies (11).

Chemotaxis assays. Neutrophils (2.3×10^6/ml) suspended in Gey medium (M.A. Bioproducts, Walkersville, Md.) with 2% bovine serum albumin were placed in modified Boyden chambers above the micropore filters (3 μm mean pore diameter, 180-μm thick cellulose nitrate filters, Sartorius Membranfilter, Göttingen, West Germany). Stimulants placed below filters included *Rhizopus* or zymosan-activated human serum, supernatants from growing or killed *R. oryzae* hyphae, *N*-formyl-methionyl-leucyl-phenylalanine (f-met-leu-phe, Sigma Chemical Co., St. Louis, Mo.), or controls (unactivated heated serum or Gey medium). Activated sera were prepared by incubation of either 0.5 mg of zymosan per ml or graded increments of *R. oryzae* hyphae with fresh human sera for 1 h at 37°C, followed by inactivation of residual complement by heating at 56°C for 30 min, removal of particles by centrifugation at 550 × *g* for 20 min at 4°C, and dilution to 5% final concentration in gelatin-Veronal-buffered saline (calcium 7.0×10^{-5} M, magnesium 5.0×10^{-4} M); control sera were incubated in the absence of activator and diluted comparably. After incubation for 30 to 45 min at 37°C, filters were removed, fixed in methanol, stained with Mayer hematoxylin, dehydrated with increasing concentrations of ethanol, cleared with xylene, and mounted on glass slides with immersion oil (10). The leading front technique of Zigmond and Hirsch (26) was then used, determining the migration front by measuring the micrometers from the top of one filter to the farthest distance traveled by two cells per high-power field. Five fields were counted per filter, and results from at least duplicate (or quadruplicate where cell numbers permitted) filters in each experiment were pooled, as the variance of migration fronts within a single filter was greater than the variance among duplicate filters (10). In selected experiments, twofold serial dilutions of *Rhizopus* supernatants were added to chemotactic chambers above and below filters for a "checkerboard" assay (26) to determine whether the stimulant induced directed migration (chemotaxis) or only increased random migration. Based upon observed neutrophil migration in increasing concentrations of supernatants in the absence of a gradient (equal concentrations above and below filters), the formula of Zigmond and Hirsch (26) was used to calculate predicted values for randomly migrating cells for each combination of supernatant concentrations above and below filters. In some experiments in which zymosan-activated serum was used as the chemotactic stimulant, antibodies to human C3 or C5 (Meloy Laboratories Inc., Springfield, Va.) were added to stimulants placed in chambers below filters.

Chemicals. Glucose, acetoacetic acid, and β-hydroxybutyric acid were obtained from Sigma.

Statistics. Means and standard errors of means for groups of data were compared by using the two-sample Student *t* test, or the paired-sample *t* test where appropriate. In other studies, relationships between groups of data were determined by calculation of correlation coefficients and testing for linearity and significance of correlation, and by comparing differences between correlation coefficients derived from two groups of data (24).

RESULTS

Hyphal metabolic activity and damage by neutrophils. Hyphae were incubated in Hanks balanced salt solution or serum (10 to 80%) for 1 h,

TABLE 1. Effects of glucose, ketoacids, and pH on migration of normal neutrophils

Alterations and additions to media				Neutrophil migration distance (μm) with the following stimulant below filter[a]:		
Glucose (mg/dl)	pH	β-OH-butyric acid (M)	Acetoacetic acid (M)	None	Zymosan-activated serum	f-met-leu-phe (5×10^{-8} M)
100	7.4	0	0	41.8 ± 5.3	125.7 ± 9.1	108.1 ± 7.1
500	7.4	0	0	40.0 ± 2.4	138.8 ± 6.0	124.0 ± 8.0
100	7.0	0	0	44.8 ± 2.8	118.2 ± 7.7	Not done
500	7.0	0	0	38.6 ± 1.5	113.9 ± 8.1	124.8 ± 7.2
100	7.4	8×10^{-3}	2×10^{-3}	38.9 ± 2.1	96.3 ± 7.7	Not done
100	7.0	8×10^{-3}	2×10^{-3}	45.0 ± 2.6	115.6 ± 7.9	Not done
500	7.4	8×10^{-3}	2×10^{-3}	36.9 ± 1.7	89.1 ± 9.6	Not done
500	7.0	8×10^{-3}	2×10^{-3}	34.3 ± 3.8	90.1[b] ± 9.3	85.9[b] ± 8.4

[a] Mean ± standard error of the mean of three separate experiments.
[b] $P < 0.05$ by one-tailed, two-sample t test.

washed, and placed in medium containing [^{14}C]uracil. In the absence of neutrophils, hyphae incubated in serum incorporated less ^{14}C than hyphae that had been incubated in buffer alone ($P < 0.01$ in 10 experiments), and this metabolic inhibition was concentration dependent: 21.7% with 10% serum, 31.2% with 30% serum, 49.1% with 50% serum, and 51.2% with 80% serum. Though inhibition appeared to be maximal with 50% serum, 80% serum was used in most subsequent experiments, first because it would provide conditions closer to those existing in vivo, and second because our studies showed that it provided maximum stability of pH during incubation when compared with low concentrations of sera or buffered medium alone. Serum-mediated inhibitory effects on hyphal metabolism were reversible: hyphae washed after incubation in serum and then reincubated in buffer in the absence of serum increased their metabolic activity toward values obtained with controls incubated in serum-free media. Neither heating serum for 30 min at 56°C nor addition of iron salts to saturate transferrin (10 μM ferric chloride or ferric ammonium sulfate) affected this inhibition of metabolic activity. Similarly, uptake of [^{14}C]uracil by hyphae was not significantly altered by glucose concentrations between 100 and 1,000 mg/dl, as uptake increased only 5.7% at the highest versus the lowest concentration. Lowering the pH of serum in incubations to 7.0 reduced serum inhibition of uracil uptake to 36.6%, compared with 53.3% inhibition in controls incubated at pH 7.4 in 10 experiments; likewise, adding ketoacids (8×10^{-3} M β-hydroxybutyric acid plus 2×10^{-3} M acetoacetic acid) at pH 7.4 reduced serum-induced inhibition of hyphal metabolism to 37.2%. Neither of these changes induced by ketoacids or lowered pH alone was statistically significant. However, supplementation of acidified (pH 7.0) sera with the above concentration of ketoacids (10^{-3} M total) significantly increased hyphal uptake of [^{14}C]uracil (i.e., serum-mediated inhibition of hyphal metabolism was reduced to 22.5%, $P < 0.05$ in four experiments), irrespective of the glucose concentration (above 100 mg/dl), thereby partially negating the inhibitory effects induced by untreated sera. After dialysis of serum against 20 volumes of saline, 76% of inhibitory activity was recovered in the dialysates. As judged by neutrophil-induced reduction in [^{14}C]uracil uptake (compared with controls which contained media with comparable concentrations of serum), the metabolic activity of hyphae was inhibited beyond effects attributable to serum alone. Moreover, in contrast to the reversible effects of serum on hyphal metabolism noted above, neutrophils appeared to damage hyphae irreversibly. Whereas 80% serum reversibly reduced uptake of [^{14}C]uracil by *Rhizopus* hyphae from 42,409 to 20,713 cpm in leukocyte-free controls, incubation with neutrophils in serum further reduced uracil uptake to 7,146 cpm, and hyphae that were washed after exposure to neutrophils did not significantly increase their metabolic activity upon reincubation in serum-free buffered medium.

Damage to hyphae by neutrophils incubated together in 80% serum was not significantly changed by combined hyperglycemia and ketoacidosis (63.2% damage at pH 7.0 in 1,000 mg of glucose per dl versus 65.5% in controls incubated at pH 7.4 in 100 mg of glucose per dl). However, hyperglycemia (final concentration of glucose 1,000 mg/dl) combined with both ketoacidosis (10^{-2} M) and lowered pH (7.0) significantly inhibited neutrophil-mediated hyphal damage (only 18.9% inhibition in three separate experiments, compared with 67.7% in control incubations performed at pH 7.4 with 100 mg of glucose per dl and no added ketoacids). Neutrophils with autologous sera from two patients with diabetic ketoacidosis (pH 7.20 and 7.28, blood glucose

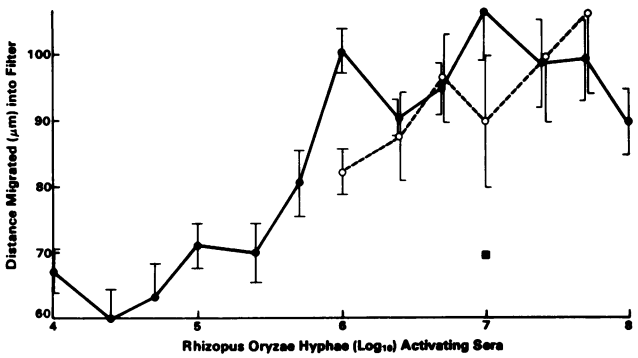

FIG. 1. Neutrophil migration determined by leading front assay stimulated by serum which had been activated by incubation of 10^4 to 10^8 *R. oryzae* hyphae. Each point denotes the mean, and brackets indicate the standard error of the mean, of 12 separate experiments involving neutrophils and autologous sera from normal subjects (●) and 5 comparable experiments with neutrophils and autologous sera from diabetic subjects (○). Results after the addition of antibodies to human C5 to activated (10^7 hyphae) normal sera (■) are shown ($P < 0.05$ by paired-sample t test in each of 2 experiments performed in which quadruplicate filters were counted).

491 to 538 mg/dl) damaged hyphae as effectively as normal neutrophils and sera (79.0 and 77.7% damage by neutrophils from the diabetic subjects compared with 75.0 and 51.3% damage, respectively, by neutrophils from two normal volunteer subjects tested simultaneously).

Effects of hyperglycemia and ketoacidosis on neutrophil chemotaxis. Hyperglycemia and ketoacidosis did not significantly affect neutrophil chemotaxis stimulated either by zymosan-activated serum or the synthetic peptide, f-met-leu-phe, in the absence of serum, though elevated concentrations of both glucose and ketoacids together marginally depressed neutrophil migration (Table 1). Results in two experiments in which 1,000 mg of glucose per dl was used in media were comparable to these results in which 500 mg/dl was used. Equivalent stimulation of migration also was seen when sera were used which had been activated by zymosan in conditions mimicking diabetic ketoacidosis, i.e., adjustment of sera to contain 500 mg of glucose per dl and 10^{-2} M ketoacids at pH 7.0.

Generation of chemotactic factors by *R. oryzae* hyphae. Fresh human sera activated by the addition of 10^6 to 10^8 *R. oryzae* hyphae stimulated neutrophil chemotaxis from a base-line, unstimulated control migration of 67.3 μm to a maximum stimulated response of 107 μm migration; this response was neutralized by the addition of antibodies to human C5 but not by antibodies to C3 (Fig. 1). Additional experiments suggested that the hyphae activated the alternative complement pathway, as heating sera at either 56 or 50°C for 30 min blocked generation of chemotactic activity, as did incubation in 0.01 M EDTA but not 0.1 M magnesium–ethyleneglycol-bis(β-aminoethyl ether)-*N*-*N*-tetraacetic acid. Moreover, *Rhizopus* hyphae generated chemotactic factors in human sera which were completely deficient in the C2 component of the classical complement pathway. Results of experiments with sera and cells from diabetic subjects were comparable to those with sera and cells from healthy, normal subjects (Fig. 1). Not shown, addition of glucose (500 mg/dl) and ketoacids (10^{-2} M) at pH 7.0 changed neither generation of chemotactic factors from sera nor the chemotactic response of neutrophils to activated sera.

Supernatants derived from live or killed (by UV light) *R. oryzae* hyphae also stimulated neutrophil migration, but did so irrespective of

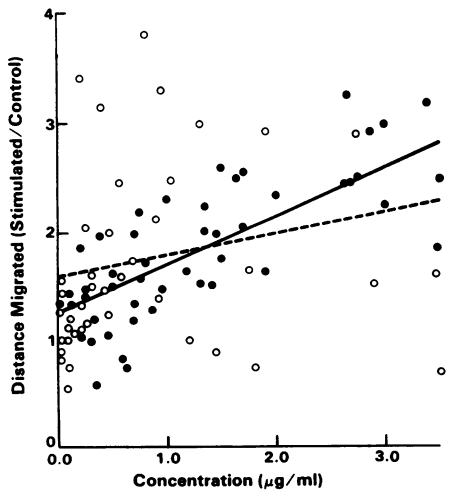

FIG. 2. Correlation of protein (●) and carbohydrate (○) concentrations of *R. oryzae* supernatants with induced neutrophil migration (shown as the ratio of distance migrated in stimulated to unstimulated control filters).

TABLE 2. Effect of variation in gradient of *Rhizopus* supernatants on neutrophil migration

Expt 1					Expt 2				
Concn of supernatant (μg/ml of protein) above filter	Distance migrated (μm) for the following concn of supernatant (μg/ml of protein) below filter:				Concn of supernatant (μg/ml of protein) above filter	Distance migrated (μm) for the following concn of supernatant (μg/ml of protein) below filter:			
	0.0	0.25	0.5	1.0		0.0	0.4	0.8	1.6
0.0	39.7[a]	60.0[b,c] (40.8)	68.2[c] (42.1)	92.0[c] (42.3)	0.0	36.2[a]	38.4 (36.0)	50.6[c] (36.1)	80.9[c] (39.3)
0.25		41.8[a]	64.1[c] (43.7)	95.7[c] (43.9)	0.4		35.9[a]	46.2 (38.0)	72.7[c] (38.3)
0.5		41.5 (43.1)	45.0[a]	71.7[c] (46.8)	0.8		36.6 (38.5)	40.2[a]	70.4 (43.9)
1.0		47.9 (51.5)	41.9 (51.7)	55.1[a]	1.6		39.1 (49.3)	42.7 (48.5)	52.2[a]

[a] Mean distances migrated (10 to 20 determinations from 2 to 4 filters in each experiment) in the absence of a gradient (equal concentrations of supernatants above and below filters).

[b] All remaining data represent means of observed migration (predicted values attributable to random migration alone calculated from observed migration in the absence of a gradient according to the formula of Zigmond and Hirsch [26]).

[c] Observed migration was greater than predicted random migration, $P < 0.05$ by two-tailed paired-sample t test.

whether serum was present. In 27 experiments performed in the absence of serum, using normal neutrophils, base-line control migration in buffer alone was 41.0 ± 2.6 μm (mean ± standard error of the mean); undiluted supernatants from killed hyphae placed in chemotactic chambers below filters resulted in an increase in migration to 97.9 ± 4.2 μm ($P < 0.0001$). These supernatants similarly stimulated increased migration of neutrophils from three different diabetic subjects (46.3 ± 5.8 μm in buffer controls, increased to 71.1 ± 5.1 μm with supernatants, $P < 0.05$). This response by diabetic neutrophils was lower than that of normal neutrophils ($P < 0.05$) when results for diabetic subjects were compared either with the entire group of 27 normal subjects by two-tailed two-sample t test, or by two-tailed paired sample t test, using results obtained from the group of three normal control subjects tested simultaneously with the diabetic cells. Though hyphae were washed repeatedly at several steps before preparation of supernatants, the possibility existed that substances from culture media were transferred with the organisms, contributing to effects on leukocyte migration. However, supernatants produced by hyphae grown in defined, synthetic media (supplemented Eagle or yeast nitrogen base) instead of Sabouraud agar and broth were similarly active in stimulating neutrophil migration, though growth of hyphae was more uniform and consistent in Sabouraud medium. Like killed hyphae, supernatants from live hyphae also stimulated neutrophil migration, but only after a two- to fourfold increase in concentration of the inhibitory material.

The ability of *R. oryzae* supernatants to stimulate neutrophil migration correlated with the protein concentration ($r = 0.7008$, $P < 0.001$) but not the carbohydrate concentration ($r = 0.2223$, $P > 0.10$) of samples, and these correlation coefficients were significantly different ($P < 0.001$) (Fig. 2).

A "checkerboard" assay, using graded concentrations of supernatant protein above and below filters, was performed to test whether the induced neutrophil migration was random or directed. Where a gradient was established by concentrations of supernatants below filters exceeding those above filters, neutrophil migration was greater than calculated predicted values attributable to random migration alone, using all six of the combinations of supernatant concentrations tested in one experiment, and four of six combinations tested in a second experiment (Table 2). In contrast, negative gradients of supernatants did not stimulate neutrophil migration.

DISCUSSION

As noted above, neutrophils appear to have a major role in host defenses against mucormycosis (6, 7, 11, 16, 17), and mobilization and migration of neutrophils to foci of inflammation are often impaired in patients with diabetes mellitus, especially when ketoacidosis ensues (8, 15, 18, 19, 22). That such a defect appears to exist in experimental mucormycosis (6, 7) makes it especially important to understand the mechanisms by which invasive hyphae generate chemotactic factors for neutrophils, as well as the effects of hyperglycemia and ketoacidosis on the process. We found that *R. oryzae* hyphae generated a chemotactic factor equally well from normal and diabetic sera. Absence of a chemotactic response to sera which had been heated before exposure to hyphae, and neutralization of the response by antibodies to C5 (but not C3), suggest that C5a was the factor generated. Thus, like many other fungi and bacteria, *Rhizopus* appears to have the capacity to activate comple-

ment by the alternative pathway. Neutrophils from normal and diabetic subjects responded comparably to this chemotactic stimulus.

R. oryzae hyphae also could generate one or more substances which stimulated directed migration of neutrophils in the absence of serum. The chemotactic activity correlated with concentration of protein but not carbohydrate, but the specific composition of the factor(s) involved remains to be determined.

Hyperglycemia and ketoacidosis may affect a variety of functions relevant to host defense mechanisms against mucormycosis (16). We observed that heated or fresh normal human serum suppressed the metabolic activity of *R. oryzae* hyphae, but this effect was negated by simulation of severe ketoacidosis. These data are consistent with the observations of Gale and Welch on inhibition of growth of *Rhizopus* spores by an uncharacterized heat-stable, dialyzable substance present in normal sera but neutralized by ketoacidosis (14); their results with spores are now extended to hyphae. Moreover, our findings indicate that inhibition by serum appears to represent a reversible fungal metabolism rather than permanent damage, at least under the conditions tested. These effects on metabolism of hyphae may be related in part to favorable conditions for growth at a more acidic pH (14, 16) and to the presence of a ketoreductase in fungi which cause mucormycosis (23), allowing them to utilize ketone bodies in their metabolism.

In contrast to serum-mediated alterations in hyphal metabolism, factors commonly associated with diabetes mellitus did not consistently affect neutrophil function. Despite the frequently observed diabetes-associated deficits in phagocytosis or intracellular killing of bacteria (2, 3, 9, 13, 21, 25), hyperglycemia and ketoacidosis did not affect surface-mediated nonphagocytic damage to hyphae by neutrophils in our in vitro assay system. However, somewhat different mechanisms may be involved in attachment to and surface killing of hyphae versus complete ingestion and intracellular killing of bacteria, and disparate results have been reported by the different laboratories studying phagocytic and bactericidal activity associated with diabetic states of varying severity. Moreover, acute bathing of normal neutrophils in media simulating abnormal conditions may not induce defects comparable to those seen after the chronic exposure of cells occurring in vivo in diabetics, and intrinsic defects in the neutrophils of diabetics may occur as well. The diabetic subjects that we were able to study were nonketotic, or at least not severely ketoacidotic, so neutrophil defects might have been observed had more seriously ill patients been tested.

Combined hyperglycemia and ketoacidosis also did not affect generation of chemotactic factors from serum. As for direct effects on neutrophils, neither lowered pH nor elevated concentrations of glucose or ketoacids individually affected results, but chemotaxis may have been marginally inhibited by the combined elevation of both glucose and ketoacids. This small effect requires further study. Neutrophils from diabetic subjects migrated significantly in response to *Rhizopus* supernatants, but there was a small but significant depression in chemotaxis relative to the activity of neutrophils from normal subjects. However, the potential biological significance of this small difference should not be overemphasized, as only three patients were studied, and comparisons were made only with cells from healthy normal subjects rather than hospitalized, comparably ill nondiabetic subjects. Further experiments will be required to settle this question.

In any case, our studies have shown that *R. oryzae* hyphae can generate chemotactic factors for neutrophils directly and by activation of serum. Occurrence of this in vivo would modulate the local response to mucormycosis in host tissues. Once neutrophils arrive in inflammatory foci, activity of antihyphal mechanisms may then be critical, in conjunction with serum factors which affect hyphal growth and influence the interactions between hyphae and neutrophils. Our data suggest that at least severe hyperglycemia and ketoacidosis alter responses of host neutrophils to *Rhizopus* hyphae in addition to directly affecting hyphal metabolism; lesser abnormalities associated with diabetes mellitus in vivo, and factors yet to be determined other than hyperglycemia and ketoacidosis, may also prove to alter host responses.

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